GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: 1/8/81

Project Title: Synthetic Protease Inhibitors

Project No: G-33-F05

Project Director: Dr. J. C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung & Blood Institute
Bethesda, MD 20014

Agreement Period: From 9/1/80 Until ~~8/31/81~~ 2/28/82 (06 Year)

Type Agreement: Grant #5R01 HL18679-06

Amount: $54,599 New PHS Funds (G-33-F05)
3,682 GIT Contribution (G-33-332)
$58,281 Total

Reports Required: Annual Progress Reports with Continuation Applications
Terminal Progress Report upon Grant Expiration

Sponsor Contact Person (s):

Technical Matters

Program Contact:
Dr. R. Sohn
301/496-7332

Program Official:
Claude Lenfant, M. D.
Director

Division of Lung Diseases
Nat'l Heart, Lung, & Blood Institute
Bethesda, MD 20014

Contractual Matters
(thru OCA)

Grants Management Contact:
Ms. Diane Grasso
301/496-7255

Grants Management Official:
James M. Pike
Chief

Grants Operation Branch
Division of Extramural Affairs
Nat'l Heart, Lung, & Blood Institute
Bethesda, MD 20014

NOTE: FOLLOW-ON TO PROJECT G-33-F04 (05 Year)

Defense Priority Rating: None

Assigned to: Chemistry (School/~~Laboratory~~)

COPIES TO:

Project Director
Division Chief (EES)
School/Laboratory Director
Dean/Director—EES
Accounting Office
Procurement Office
Security Coordinator (OCA)
~~Reports Coordinator (OCA)~~

Library, Technical Reports Section
EES Information Office
EES Reports & Procedures
Project File (OCA)
Project Code (GTRI)
Other C. E. Smith

CA—3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

SRP500
2-F
10-F

Date 10/18/82

Project Title: Synthetic Protease Inhibitors

Project No: G-33-F05

Project Director: Dr. J. C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung & Blood Institute
Bethesda, MD 20014

Effective Termination Date: 2/28/82 (06 year)

Clearance of Accounting Charges: ----

Grant/Contract Closeout Actions Remaining:

NONE

- ☐ Final Invoice and Closing Documents
- ☐ Final Fiscal Report
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☐ Other _____

Assigned to: Chemistry (School/~~Laboratory~~)

COPIES TO:

RAN
~~Administrative Coordinator~~
Research Property Management
Accounting
Procurement/EES Supply Services
Research Security Services
~~Reports Coordinator (OCA)~~
Legal Services (OCA)
Library
EES Public Relations (2)
Computer Input
Project File
Other GTRI

Synthetic Protease Inhibitors

5 R01 HL 18679-06

G-33-F05

Final Progress Report

by

James C. Powers
School of Chemistry
Georgia Institute of Tech.
Atlanta, GA 30332
(404) 894-4038

June 1982

Period Covered

9/1/75 through 2/28/82

Publications Since Last Progress Report

"Substrate Specificity of Two Chymotrypsin-like Proteases from Rat Mast Cells Studies with Peptide 4-Nitroanilides and Comparison with Cathepsin G," N. Yoshida, M. T. Everitt, H. Neurath, R. G. Woodbury and J. C. Powers (1980) Biochemistry 19, 5799-5804.

"Proteolytic Events in Replication of Animal Viruses," B. D. Korant, N. L. Chow, M. O. Lively and J. C. Powers (1980) Ann. N. Y. Acad. Sci. 343, 304-318.

"Protease Activities Present in Dog Pancreatic Membranes that Process Human Pre-Placental Lactogen," M. Zimmerman, B. M. Ashe, A. W. Alberts, P. A. Pierzchala, J. C. Powers, N. Nishino, A. W. Strauss and R. A. Mumford (1980) Ann. N. Y. Acad. Sci. 343, 405-515.

"Human Leucocyte Elastase and Cathepsin G: Structural and Functional Chracteristics," J. Travis, P. J. Giles, L. Porcelli, C. F. Reilly, R. Baugh and J. Powers (1980) Protein Degradation in Health and Disease (Ciba Foundation Symposium 75) pp. 51-68.

"Limited Proteolysis and Viral Replication," B. D. Korant, M. O. Lively and J. C. Powers (1980) Biochem. Soc. Trans. 8, 417-419.

"Protein Synthesis and Cleavage in Picornavirus-Infected Cells," B. D. Korant, J. Langner, and J. C. Powers (1980) Biosynthesis, Modification, and Processing of Cellular and Viral Polypeptides (G. Koch and D. Richter, Eds.) pp. 277-288, Academic Press, N. Y.

"Effects of Oligopeptide Chloromethylketone Administered after Elastase-Renal Toxicity and Lack of Prevention of Experimental Emphysema", V. Ranga, J. Kleinerman, M.P.C. Ip, J. Sorensen and J. C. Powers (1981) Am. Rev. of Respiratory Dis. 124, 613-618.

"The Moderation of Elastase-Induced Emphysema in the Hamster by Intratracheal Pretreatment or Post-treatment with Succinyl Alanyl Alanyl Prolyl Valine Chloromethyl Ketone," P. J. Stone, E. C. Lucey, J. D. Calore, G. L. Snider, C. Franzblau, M. J. Castillo, and J. C. Powers (1981) Am. Rev. Respir. Dis. 124, 56-59.

"The Effects of Small Doses of Oligopeptide Elastase Inhibitors on Elastase-Induced Emphysema in Hamsters - A Dose-Response Study", M.P.C. Ip, J. Kleinerman, V. Ranga, J. Sorensen, and J. C. Powers (1981) Am. Rev of Respiratory Dis. 124, 714-717.

"Synthetic Elastase Inhibitors and Their Role in the Treatment of Disease," J. C. Powers, A. Yasutake, N. Nishino, B. F. Gupton, and C-M. Kam (1981) Peptides (D. Rich and E. Gross, Eds.) pp 391-399, Pierce Chem. Co., Ill.

"Proteolytic Enzymes and Their Active-Site Specific Inhibitors: Role in the Treatment of Disease," J. C. Powers (1982) Modification of Proteins. Food Nutritional, and Pharmacological Aspects. Adv. in Chemistry Series 1981 (R. E. Feeney and J. R. Whitaker, Eds.) pp 347-367, Am. Chem. Soc., Wash., D.C.

"Specificity and Reactivity of Human Leukocyte Elastase, Porcine Pancreatic Elastase, Human Granulocyte Cathepsin G, and Bovine Pancreatic Chymotrypsin with Arylsulfonyl Fluorides. Discovery of a New Series of Potent and Specific Irreversible Elastase Inhibitors", T. Yoshimura, L. N. Barker, and J. C. Powers (1982) J. Biol. Chem. 257, 5077-5084.

"A New Class of Heterocyclic Serine Protease Inhibitors. Inhibition of Human Leukocyte Elastase, Porcine Pancreatic Elastase, Cathepsin G, and Bovine Chymotrypsin A with Substituted Benzoxazinones, and Anthranilates", T. Teshima, J. C. Griffin, and J. C. Powers (1982) J. Biol. Chem. 257, 5085-5091.

Reprints which have not previously been submitted are included. Reprints for several recent papers have not yet been received.

Progress Report

Scientific Goals. A number of proteases (protein hydrolyzing enzymes) have been shown to be involved in diseases such as pulmonary emphysema and arthritis which involve tissue destruction. The goal of this research was to design and synthesize specific and effective protease inhibitors. The inhibitors should be invaluable in the study of the normal biological function and the role of proteases in disease. In addition, synthetic protease inhibitors should find use in the clinical treatment of a variety of diseases including pulmonary emphysema, rheumatoid arthritis, certain types of tumors and viral infections.

Significance. Proteases are involved in the regulation of many important biological processes. Some of the better known examples are blood coagulation, fibrinolysis, complement activation, fertilization, protein processing, digestion and phagocytosis. Some of these processes involve cascades of proteolytic events. For example, in the resting state, both the coagulation and complement systems are composed of a number of plasma zymogens of proteases. Upon receipt of an appropriate signal some of these zymogens are converted into active proteases which in turn activate other zymogens producing a cascade of enzymatic reactions. The initial signal in the coagulation system is the formation of new surfaces at the site of a wound and the end result is formation of the fibrin clot. In the complement system, initial formation of antigen-antibody complexes leads to the lysis of foreign cells. In both cases, the cascade of proteolytic events gives a system for prompt amplification and control of the initial signal.

Proteases and Disease. Since a major component of all cells is protein, proteases could be very destructive if they were not carefully controlled or compartmentalized. The potential seriousness of uncontrolled proteolysis can be recognized by the fact that ca. 10% of the proteins by weight found in human plasma are protease inhibitors. In addition to the plasma inhibitors, there are other inhibitors which are more localized and have not been as well characterized. It is now believed that many diseases result when there is an imbalance between specific proteases and their natural inhibitors. Some examples of proteases which have been linked to specific diseases (and the protease involved) are emphysema (leukocyte elastase and cathepsin G), arthritis (collagenase), pancreatitis (pancreatic serine proteases), cancer (collagenase, plasminogen activator), hypertension (renin and angiotension converting enzyme), inflammation (mast cell proteases) and amyloidosis (elastase).

Emphysema. Emphysema is one disease where the linkage between proteolysis and the disease is fairly well understood. Pulmonary emphysema is a disease characterized by a progressive loss of lung elasticity due to the destruction of lung elastin and alveoli. Respiration becomes increasingly difficult and death often results.

The first clue to the involvement of proteases in emphysema was the observation by Laurell and Ericksson that individuals who were homozygotes in an α_1-protease inhibitor (α_1-antitrypsin) deficiency were predisposed to the disease. This serum protease inhibitor inhibits a spectrum of proteases including elastase and cathepsin G which are found in the granules of human

leukocytes. Leukocyte proteases are normally involved in phagocytosis in the lung and contribute to the turnover of damaged lung cells and the digestion of invading bacteria. In normal individuals α_1-PI protects the lung from digestion of any of the proteases which may leak from leukocytes. In individuals not so protected, proteolysis of lung elastin leads to the development of emphysema. The α_1-PI of PiZ individuals has a one amino acid substitution (Lys for Glu) somewhere in the sequence. As a result, the α_1-PI of PiZ individuals accumulates in the liver and is not exported to the plasma.

A major predisposing factor for the development of chronic obstructive pulmonary disease is cigarette smoking. It is now believed that α_1-PI is inactivated either directly by oxidants in the smoke or by myeloperoxidase which is released from leukocytes.

Cigarette smoke decreases the α_1-PI activity in rat lung and produces a functional deficiency of protease inhibitors in the lower respiratory tract of humans. It is quite reasonable to assume that this is due to the oxidation of the essential methionine residue in α_1-PI. Thus, chemical modification of α_1-PI by oxidation of a methionine residue to a methionine sulfoxide residue by some component of cigarette smoke or by myeloperoxidase which is released by cigarette smoke, results in inactivation of this essential protease inhibitor. The resulting imbalance of proteases and protease inhibitors in the lung then results in the development of emphysema.

Synthetic Protease Inhibitors. Our research on synthetic protease inhibitors has been focused on two of the four classes of proteases. One major goal was to develop specific and effective inhibitors for the serine proteases elastase and cathepsin G from human clinical treatment of pulmonary emphysema. The second major goal was to develop new types of inhibitors for metalloproteases. Initially we utilized thermolysin as a model system for the development of new classes of inhibitors. We then tried to extend these inhibitors to other important metalloproteases.

Studies with Human Leukocyte Enzymes. Proteolysis by enzymes released from human PMN leukocytes are involved in several major diseases which involve tissue destruction. In the case of pulmonary emphysema, elastase seems to be principally responsible for lung damage with cathepsin G and other protease carrying out secondary digestions. We have previously synthesized a number of specific peptide chloromethyl ketone inhibitors of both human leukocyte elastase and cathepsin G. The best elastase inhibitor is MeO-Suc-Ala-Ala-Pro-ValCH_2Cl and the best cathepsin G inhibitor is Z-Gly-Leu-Phe-CH_2Cl.

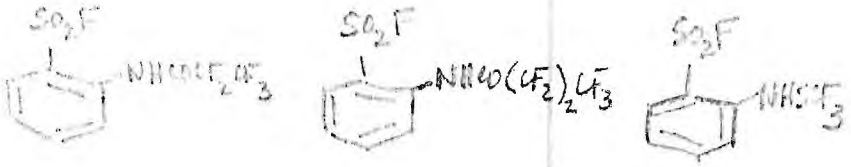
One concern that is often expressed about peptide chloromethyl ketones, is their ability to alkylate a variety of nucleophiles in proteins. This is thought to be a major limitation to the use of chloromethyl ketones in physiological situations. In order to evaluate the significance of side reactions with other nucleophiles, the rate of reaction of the thiol glutathione with MeO-Suc-Ala-Ala-Pro-ValCH_2Cl was measured and found to be quite slow. This inhibitor would discriminate in favor of leukocyte glutathione by a factor of 1770 if the concentrations were equivalent. Thus, MeO-Suc-Ala-Ala-Pro-ValCH_2Cl appears to be a highly reactive and selective elastase inhibitor for use in physiological situations.

Animal Studies. Three research groups have studied the effect of synthetic elastase inhibitors in animal models of emphysema. We collaborated with and supplied inhibitors to all three groups. In each case, elastase was used to induce emphysema in the lungs of hamsters or mice. Dr. J. Kleinerman showed that Ac-Ala-Ala-Pro-AlaCH_2Cl had significant anti-elastase activity in vivo (hamsters), markedly decreased the extent of elastase-induced emphysema, and produced no adverse toxic effects during the period covered by the study (120 days). Dr. P. Stone (Boston U.) has obtained similar results using Suc-Ala-Ala-Pro-ValCH_2Cl. Dr. A. Janoff (Stony Brook) has shown that MeO-Suc-Ala-Ala-Pro-ValCH_2Cl is orally active in preventing elastase induced emphysema in mice.

We are continuing to collaborate with the research groups carrying out animal studies with elastase inhibitors. We completed the synthesis of 9 g of Ac-Ala-Ala-Pro-AlaCH_2Cl for Dr. Kleinerman. He is currently studying the effects of this inhibitor on the progression of emphysema in hamsters. During a two year study, the inhibitor will be given daily subsequent to induction of emphysema in the animals.

At present many questions remain concerning the toxicity, immunogenicity and carcinogenicity of peptide chloromethyl ketones. However, these inhibitors have already been useful in showing that elastase inhibitors can be used for the in vivo treatment of emphysema.

Sulfonyl Fluorides. Sulfonyl fluorides inhibit serine proteases by reaction with the active site serine residue. Previously we investigated the rates of inhibition of elastase and cathepsin G by a variety of sulfonyl fluorides and found relatively little selectively or reactivity. However, we recently discovered that the introduction of fluoroacyl groups into the sulfonyl fluoride structure gives inhibitors with considerable reactivity and selectivity.

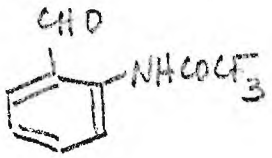


Human Leukocyte Elastase	1700	1400	1200
Porcine Pancreatic Elastase	1300	62	26
Cathepsin G	13	5.8	13

The values listed under each compound are inhibition rates ($k_{obs}/[I]$ at pH 7.5). These are the best elastase inhibitors of a large number of sulfonyl fluorides which we have synthesized and tested. All three are very effective inhibitors of human leukocyte elastase and the latter two are quite specific. Sulfonyl fluorides without the fluoroalkyl groups or the meta- and para- isomers are unreactive. Sulfonyl fluorides are not generally considered to be toxic and these inhibitors may find utility in the treatment of human disease.

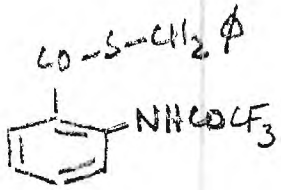
This work has recently been published in detail (Yoshimura et al., 1982).

Other Inhibitor Structures. Based on our work with sulfonyl fluoride inhibitors of HL elastase, we reasoned that other structures containing fluoroacyl groups might be elastase inhibitors. In particular, the aldehyde 1 might be a transition state analog and form a hemiacetal with the enzyme. The nitrophenyl ester 2 and the thiobenzyl ester 3 might be substrates or specifically acylate the enzyme and form stable acyl enzyme derivatives. Thus we decided to synthesize these compounds.



1

2



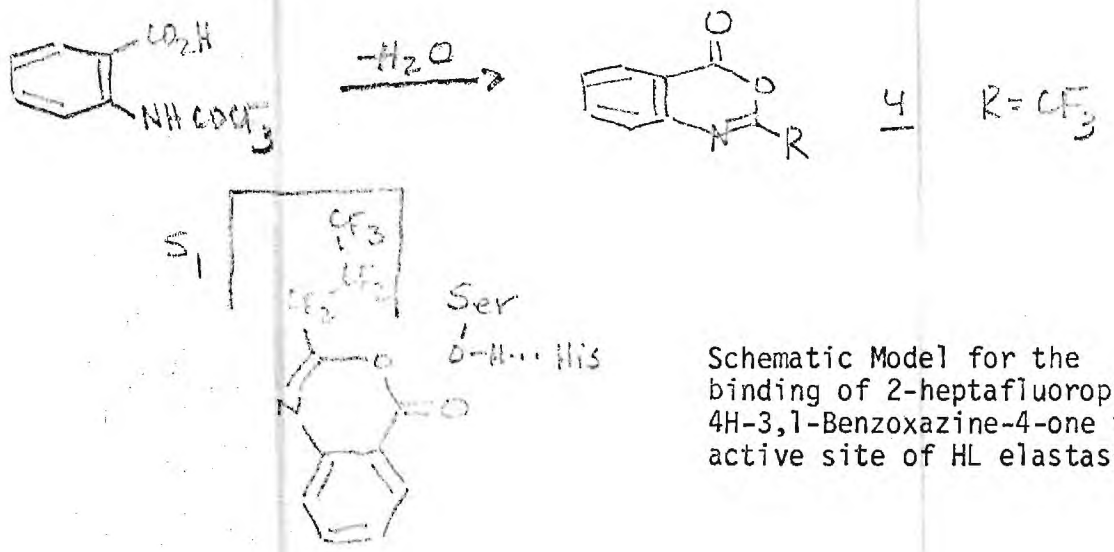
All of the compounds reacted with elastase, although not necessarily in the way that we had expected. The aldehyde was a weak inhibitor with a K_I of 18 mM. The nitrophenyl ester was fairly unstable in aqueous medium, but did not acylate elastase. However the acyl enzyme was unstable and hydrolyzed fairly rapidly. The thiobenzyl ester in contrast was not a HL elastase substrate, but instead was a potent inhibitor with a K_I of 1 μM. Needless to say, we synthesized a number of other thioester derivatives related to 3 and discovered a fair number of potent inhibitors for elastase and chymotrypsin which I will not describe (they are discussed in Teshima et al., 1982).

HETEROCYCLIC INHIBITORS. During the synthesis of thioesters of N-acyl-anthranilic acid related to 3, we became aware of the fact that cyclization to 2-substituted-4H-3,1-Benzoxazin-4-ones (4) could take place.

We synthesized 4 and found it to be a potent elastase inhibitor. We then decided to synthesize other derivatives and found a number of benzoxazinones with K_I values in the 10^{-6} to 10^{-8} M range. The best HL elastase inhibitor was the heptafluoropropyl derivative (4, R=$CF_2CF_2CF_3$) which had a K_I of 92 nM.

The reversible HL elastase inhibitors which we discovered are significantly better than most of the previously reported reversible inhibitors for this enzyme. In fact the K_I values observed with the benzoxazinones are almost 10 fold lower than those reported for transition analogs such as Ac-Pro-Ala-Pro-NHCH(CH_3)CHO with the related enzyme, PP elastase (K_I=8.10^{-7}M, Thompson, 1973). Our best cathepsin G inhibitor (4, R=$CF_2CF_2CF_3$) was equally as potent as the peptide aldehyde chymostatin (a transition state inhibitor).

We believe the fluoroalkyl or alkyl substituents on the various heterocyclic ring systems are interacting with the S_1 pocket of HL elastase. With the benzoxazinones, the 2-propyl derivative has a lower K_I than either the benzyl or methyl derivative which is consistent with the substrate specificity of this enzyme. In all cases, substitution of a fluoroalkyl group for an alkyl group decreased the K_I value. In most cases, a one-to-two order of magnitude change was observed. Again this is consistent with binding in the hydrophobic S_1 pocket.

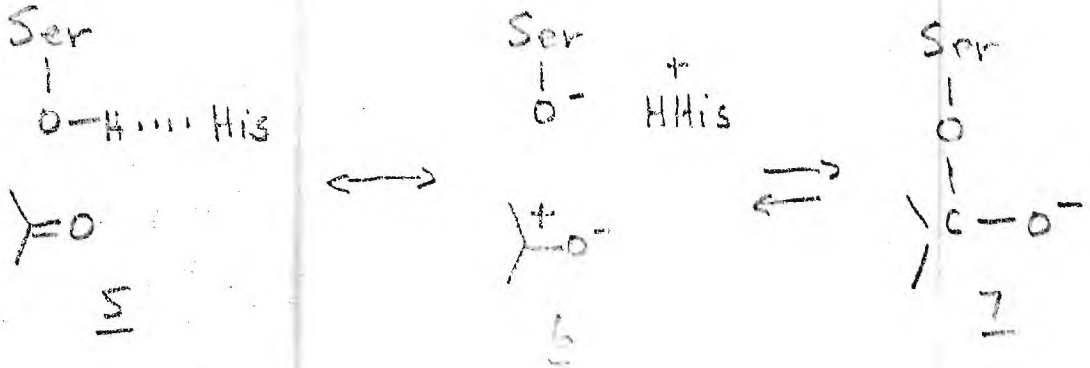


Schematic Model for the binding of 2-heptafluoropropyl-4H-3,1-Benzoxazine-4-one to the active site of HL elastase

During the course of this investigation we noticed that there seemed to be a correlation between the inhibitory activity of the various inhibitors and their carbonyl stretching frequencies in the infrared. We then made a plot of pK_I versus carbonyl stretching frequency for 26 different compounds. An excellent correlation was observed with the best inhibitors having the higher carbonyl stretching frequencies. If all 26 compounds were included, a correlation coefficient of 0.84 was obtained in a least square fit of the data. However, if only trifluoroacetyl or trifluoromethyl derivatives were included, the correlation coefficient increased to 0.89. The inhibitors studied ranged in K_I values from 10^{-2} to 10^{-7} and had carbonyl stretching frequencies that had a range of 120 cm^{-1}. Thus the correlation between pK_I and the carbonyl stretching frequency is astonishingly good.

The strong correlation between the carbonyl stretching frequency and pK_I provides strong support for the hypothesis that all of the inhibitors are interacting with elastase at their carbonyl groups. As the carbonyl stretching frequency increases, binding of the inhibitors to the enzyme increases. Carbonyl compounds become more polarized and more susceptible to nucleophic attack as the carbonyl stretching frequency increases. We propose that an electrostatic interaction is occurring between the charge relay system of the serine protease and the carbonyl group of the inhibitors (5 to 6). If the serine oxygen is interacting with the carbonyl carbon as shown in 6, it is not difficult to imagine the formation of a tetrahedral intermediate if bonding occurred between the carbonyl carbon and the serine γ-oxygen (7). Such tetrahedral complexes

are formed upon interaction of peptide aldehydes with serine proteases. However if a tetrahedral complex is formed between any of our inhibitors and elastase, there must be forces which restrain further reaction to form an acyl enzyme, followed by hydrolysis. With the sole exception of the nitrophenyl ester 2, none of the inhibitors were hydrolyzed by any of the enzymes investigated. Since formation of a tetrahedral intermediate 7 without continuation on the reaction pathway seems rather improbably to us for many of the compounds examined, we prefer an inhibition model which simply involves interaction of a partially polarized carbonyl group with a partically polarized charge relay system.



5 6 7

In conclusion we have developed a novel series of potent heterocyclic inhibitors for HL elastase and other serine proteases. Some of the inhibitors are highly specific for HL elastase. The inhibitors can be considered in one sense to be transition state analogs. Also we have confirmed the observation with sulfonyl fluorides that it is feasible to develop small non-peptide molecules which are effective and specific inhibitors for HL elastase.

This work has been completely described in a recent publication (Teshima et. al., 1982).

Metalloproteases. A number of metalloproteases are involved in diseases which involve connective tissue destruction. Collagenase has been found in rheumatoid synovium and has been implicated in the destruction of joints in rheumatoid arthritis. Collagenase may also be involved in periodontal disease, corneal ulceration, and several other diseases. Invasive tumors have been shown to secrete collagenase and the ability of this enzyme to attack connective tissue may allow such tumors to expand into the surrounding tissue.

Our long-term plan for the development of collagenase inhibitors was to use thermolysin as a model system. This zinc metalloprotease is more readily available than collagenase and would bind smaller peptides. This allowed us to investigate a number of different inhibitors (both reversible and irreversible) for thermolysin (Rasnick and Powers, 1978; Nishino and Powers, 1979 reprints previously supplied). The best reversible inhibitors are phosphoramidates, hydroxamic acids and thiols. The hydroxamic acid NONH-Bzm-Ala-Gly-NH_2 (Bzm= -$COCH(CH_2C_6H_5)CO$-) is an excellent inhibitor of thermolysin (K_I = 0.7 μM) and has been attached to agarose and used in the affinity purification of thermolysin. A number of irreversible thermolysin inhibitors such as $ClCH_2CON(OH)CH(CH_2CH(CH_2CH(CH_3)_2CO_2CH_3$ were synthesized. The site of the reaction was determined.

Pseudomonas aeruginosa Elastase. Once we had developed specific inhibitors for thermolysin, we began to extend these inhibitors to other metalloproteases. One of our first targets was the elastase from P. aeruginosa since it's substrate specificity was quite similar to that of thermolysin. Pseudomonas aeruginosa elastase in an infectious organism which is resistant to many antibiotics. This organism cause hemorrhagic pneumonia in mink and corneal ulcers in man. Many strains of P. aeruginosa produce an elastase. Those strains with elastase have been shown to be more pathogenic than those without. The elastase is likely the factor responsible for the destruction of corneal tissue and hemorrages of the lung observed in P. aeruginosa infections.

We synthesized a new substrate and a number of inhibitors for P. aeruginosa elastase (Nishino and Powers, 1980). The substrate Abz-Ala-Gly-Leu-Ala-Nba (Abz, 2-aminobenzoyl; Nba, $NHCH_2H_4NO_2$) contains a fluorescent group (Abz) and a quenching group (Nba). Cleavage of the peptide by elastase results in a 6- to 7-fold increase in fluorescence. A sensitive rate assay utilizing this substrate was developed and used to study inhibitors. Some of the best reversible inhibitors are listed below:

$HONHCOCH(CH_2C_6H_5)CO$-Ala-Gly-NH_2	K_I = 0.044 μM
$HSCH_2CH(CH_2C_6H_5)CO$-Ala-Gly-NH_2	0.064 μM
CHO-HOLeu-Ala-Gly-NH_2	8.0 μM
phosphoramidon	0.055 μM

We have proposed that the inhibitors bind to the extended substrate binding region of elastase with the inhibitor ligating the active site zinc of the enzyme. A moderate irreversible inhibitor was also designed and synthesized. Such inhibitors may be of value in the future for the treatment of infections due to P. aeruginosa. This work has been reported (Nishino & Powers, 1980).

The thermolysin and P. aeruginosa elastase inhibitors are also effective at inhibiting a metalloprotease from dog pancreatic membranes (Zimmerman et. al., 1980). In addition, some of the compounds inhibit a mouse ascites tumor dipeptidase (collaborative project with E. Patterson, Fox Chase Cancer Center, Phil., Pa.). The hydroxamic acid metalloprotease inhibitors which we have developed have been extended to the angiotensin converting enzyme by two industrial groups and to aminopeptidases by two other research groups. Finally one of our hydroxamic acid thermolysin inhibitors has recently been shown to be a potent enkephalinase inhibitor.

Thus far little progress has been made toward the development of a collagenase inhibitor. We plan to pursue this problem in the future.